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ICMSF methods studies. IV. International collaborative assay for the detection of *Salmonella* in raw meat[1,2,3]

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An international collaborative assay of methods for the isolation of *Salmonella* from raw meats showed that more positive samples were identified when enrichment broth was incubated at 43C than at 35C. The choice of enrichment broth appears to be influenced by the serotype involved. Selenite brilliant green sulfa broth was the best enrichment medium for *S. schwartzengrund*, *S. typhimurium*, and *S. dublin* while selenite cystine broth was best for *S. senftenberg*. With *S. newport* and *S. worthington* no significant difference between tetrathionate broth and selenite brilliant green sulfa broth was apparent. While bismuth sulfite agar was successful for isolations from positive samples, it gave a large number of false-positive isolates when used with *Salmonella*-negative samples. This did not happen with the brilliant green agars used. Best results from selective agars were obtained when laboratories were allowed to choose their own, indicating that familiarity with a medium plays a significant role in its suitability for the isolation of *Salmonella*.

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Une évaluation faite sur le plan d'une collaboration internationale des méthodes d'isolement de *Salmonella* dans des viandes crues a montré que l'on détecte un plus grand nombre d'échantillons positifs lorsque le bouillon d'enrichissement est incubé à 43C plutôt qu'à 35C. Le choix du bouillon d'enrichissement dépend du sérotype en cause. Le bouillon selenite vert brillant sulfa est le meilleur milieu d'enrichissement pour *S. schwartzengrund*, *S. typhimurium* et *S. dublin* tandis que le bouillon selenite cystine semble le meilleur pour *S. senftenberg*. Dans le cas de *S. newport* et *S. worthington* il n'y a apparamment pas de différence significative entre le bouillon tetrathionate et le bouillon selenite vert brillant sulfa. La gélose bismuth sulfite convient très bien pour les isolements à partir de matériel positif, mais il donne un grand nombre d'isolements faussement positifs à partir d'échantillons qui ne contiennent pas de *Salmonella*. Cela ne se produit pas en utilisant la gélose vert brillant. C'est en laissant les laboratoires choisir eux-mêmes leurs milieux que l'on obtient les meilleurs résultats sur les géloses sélectives. Cela confirme que l'habitude d'un milieu de culture joue un rôle significatif dans la rentabilité d'un milieu pour l'isolement de *Salmonella*. [Traduit par le journal]

Introduction

The ICMSF text *Microorganisms in foods: their significance and methods of enumeration* (4) describes analytical methods whose tentative selection was based on a consensus of experience by the members of the Commission. More definitive expressions of preference were agreed on to require international comparative testing. The Commission proposed to publish a series of such comparative studies. The present paper reports the results of such tests designed to determine the most effective method among those currently described for the isolation of *Salmonella* from raw meats. A total of 13 laboratories from eight countries participated in comparing the recovery of six serotypes of *Salmonella* artificially inoculated into ground beef. For these tests incubation temperatures of 35C and 43C were used together with various enrichment and plating media. Two separate experiments were conducted.

Materials and Methods

Minced beef was obtained from a commercial source, passed through a fine grinder, and then mixed in bulk in a Hobart food mixer until it appeared uniform. It was then weighed into individual 25-g portions, placed in individual sterile plastic bags, and held overnight at 5C. To determine the absence of *Salmonella*, six portions from this lot were analyzed, using selenite brilliant green sulfa enrichment

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[2]International Commission for Microbiological Specifications for Foods (ICMSF) — a commission of the International Association of Microbiological Societies.

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broth at 43C. In addition the aerobic plate count (standard plate count agar at 35C) and numbers of coliforms (violet red bile (VRB) agar at 35C) were determined.

Inocula for these samples were prepared as follows. Selected serotypes of *Salmonella* were cultured in meat infusion broth at 35C for 24 h and diluted to obtain about five cells per 0.2 milliliter. The center of each 25-g portion of meat, other than control portions, was inoculated with 0.2 ml of the diluted culture by using a Cornwall syringe equipped with a two-way automatic non-return valve and a 1½ in. needle. After inoculation, the samples were held overnight at 5C and the next day the bags were heat-sealed and placed in a freezer at −25C. The frozen specimens were packed in Dry Ice in insulated shipping containers and shipped by air freight to the participating laboratories. The shipping containers had a sample cavity of 7.5 × 7.5 × 5.75 in. surrounded by 1.6 in. of styrofoam which was in turn incased in a double-layered box made of waxed corrugated cardboard. Trials had shown this shipping procedure was capable of holding samples frozen for at least 60 h at room temperature, and by prearranging shipments, it was possible to deliver samples to all participants within 24 h of packing time. Separate samples were provided for each of the methods under test.

Analytical procedures used were those given by Thatcher and Clark (4). In addition, selenite brilliant green sulfadiazine broth was also used as an enrichment medium. This was included because of our own very favorable experience with this medium at an incubation temperature of 43C. Unless otherwise noted, all media were purchased from one common supplier (Difco) and each participant received a portion from the same lot for each of the media used.

Meat for the first experiment had an aerobic plate count of 6.1×10^6 per gram, and a coliform content of 2.6×10^5 per gram. Samples were uninoculated (controls) or inoculated with *S. schwartzengrund* to a level of five cells per 25 grams meat. This level of inoculum was used to attempt to ensure that all positive samples would in fact contain *Salmonella*. Each of eight laboratories received two positive and two negative samples for each method to be tried, and two technicians working independently were asked to analyze a positive and a negative set of samples with these methods. None of the analysts were informed of the makeup of their samples.

A second experiment was conducted with participation by 13 laboratories in eight countries. *Salmonella* representing O groups B, C_2, D, E_4, and G were used. Inoculations were done as for the previous experiment with each test portion receiving only one serotype. Each analyst received three replicate packs of samples. One set of samples was tested using a common lot of medium for each of the media required; a second set was tested using the same type of media but acquired locally by the participating laboratory; and finally, for those who had a personally preferred selective agar, such "choice" was permitted in addition to those specified. The enrichment procedures were limited to tetrathionate broth at 43C, selenite brilliant green sulfa broth at both 35 and 43C, and selenite cystine broth at 43C. The use of the latter, in spite of its poor recovery in the first trial, was because of opinion expressed at a Commission meeting that perhaps recovery by selenite had been poor as a result of carryover of selenite from the enrichment culture to the selective agar, thus increasing the inhibitory power of the selective agar. Provision was therefore made for the selenite cystine broth to be used directly on the selective agars and also to be diluted 1:100 with phosphate-buffered water before plating on selective agar.

The minced beef used for the second experiment had an aerobic plate count (35C) of 1.08×10^9 per gram and a coliform (VRB agar) count of 2.85×10^5 per gram. Before inoculation, *Salmonella* was not recovered from 6 × 25 g portions. The average amounts of the test *Salmonella* strains added per 25 grams of meat was *S. typhimurium* 7.6 cells, *S. newport* 7.0 cells, *S. dublin* 7.0 cells, *S. senftenberg* 4.2 cells, and *S. worthington* 7.2 cells, as judged by quintuplicate counts of the inoculum plated on MacConkey Agar. Only one serotype was used in each inoculated sample.

Results and Discussion

The results of the analysis of the first experiment are presented in Table 1. In that section of Table 1 marked A the analysis was essentially perfect and no differentiation can be made between the enrichment–temperature–plating medium combinations represented. It is equally apparent that the use of tetrathionate enrichment at 35C or selenite cystine enrichment at either temperature was not as effective as tetrathionate enrichment at 43C or selenite brilliant green sulfa enrichment at either temperature.

Differences were apparent when laboratories failed to detect *Salmonella* in all positive samples (Table 1, section B). The data in section B of Table 1 were subjected to an analysis of variance and the result indicated that selenite brilliant green sulfa at 43C was the best enrichment medium (at 15% significance level) but that the plating media do not show significant differences in influencing the recovery. To assist in interpreting the influence of the plating medium, each analyst was asked to arbitrarily evaluate the plating media during this experiment. The evaluation scheme used is given in Table 2.

The analysts ratings were then transposed as indicated to correlate with known presence or absence of *Salmonella* in the test specimens. Thus good discrimination with both positive and negative samples would be reflected by higher values for a given plating medium. For example, if an analyst saw 25 to 50% of the colonies as suspect colonies on a given plate for a given sample he would rate that plate as 3. If this sample was known to contain *Salmonella*, it would be assigned a transposed value of 3 (desirable) but if such a plate was obtained from a

TABLE 1

Recovery of *Salmonella* from meat (positive samples)

Enrichment broth	Incubation temp., °C	Plating medium*	Laboratory 1	2	3	4	5	6	7	8
Tetrathionate	35	BG	1†	0	0	0	2	0	0	0
		BGS	0	0	0	0	1	0	1	0
		BiS	0	1	0	0	2	2	0	0
			A						B	
	43	BG	2	2	2	2	2	2	0	2
		BGS	2	2	2	2	2	2	2	1
		BiS	2	2	2	2	2	2	1	2
Selenite brilliant green sulfa	35	BG	2	2	2	2	2	2	1	1
		BGS	2	2	2	2	2	2	1	1
		BiS	2	2	2	2	2	2	1	1
	43	BG	2	2	2	1/1	2	2	1	2
		BGS	2	2	2	1/1	2	2	2	2
		BiS	2	2	2	1/1	2	2	2	2
Selenite cystine	35	BG	0	1	0	0	1	1	0	2
		BGS	1	1	0	0	0	0	1	1
		BiS	0	2	0	0	2	2	0	2
	43	BG	0	1	0	1	2	2	1	2
		BGS	0	1	0	2	2	1	0	1
		BiS	1	2	1	2	2	2	1	2

*BG, brilliant green; BGS, brilliant green sulfa; BiS, bismuth sulfite.
†There were two technicians and therefore two assays. Entries 0, 1, and 2 signify 0, ½, or 1 successful positive assays. Entries ½ signify that one assay was missing, but the one carried out was successful.

TABLE 2

Rating of performance of plating media using analysts rating of plates examined

	Analysts rating	Transposed valuation* Negative samples	Positive samples
Salmonella suspect colonies absent	1	5	−1
Suspect colonies up to 25% of all colonies	2	−2	2
Suspect colonies 25–50% of all colonies	3	−3	3
Suspect colonies 50–90% of all colonies	4	−4	4
Suspect colonies over 90% of all colonies	5	−5	5

*To obtain transposed valuations, the analysts rating was assigned a new value which took into account whether or not the sample being analyzed contained *Salmonella*.

Salmonella-negative sample, it would be assigned a transposed value of −3 (undesirable). The total scores for all plating media with all samples are presented in Table 3. It may readily be seen from this table that the selective agars were most effective after enrichment at 43C; these figures also agree with the statistical determination that use of selenite brilliant green sulfa enrichment at 43C was the best selective enrichment procedure. A point not obvious here, and which accounts for the lower rating received by bismuth sulfite agar was the frequency with which it lead to isolation of suspect colonies from the *Salmonella*-negative samples. In this test the use of bismuth sulfite agar led to a great deal of unnecessary time and effort in the case of negative samples, although its performance with positive samples could not be differentiated from the other agars used.

Freidman's two-way ranking tests (3) were applied to the data obtained from the second experiment, using the laboratories as blocks and

TABLE 3

Media scores obtained from analysis of all samples of meat (positives and negatives). Higher scores indicate better performance

Enrichment	Tetrathionate		Selenite brilliant green sulfa		Selenite cystine	
Plating medium	35C	43C	35C	43C	35C	43C
Brilliant green agar	30	105	90	130	29	67
Brilliant green sulfa agar	45	127	101	150	62	75
Bismuth sulfite agar	5	61	76	114	9	25

ranking the enrichments within the blocks. The analyses were run for each combination of serotype and method, and the rankings obtained are presented in Table 4.

For recovery of *S. senftenberg*, selenite cystine broth incubated at 43C was the best enrichment medium, and there appeared to be no advantage in diluting the enrichment culture before plating. For recovery of *S. typhimurium* and *S. dublin*, the enrichment of choice appeared to be selenite brilliant green sulfa with incubation at 43C slightly better than incubation at 35C. For *S. newport* and *S. worthington* recovery was not significantly different, regardless of the enrichment procedure used although there is a slight suggestion that the use of tetrathionate broth incubated at 43C was better, followed by selenite brilliant green sulfa broth also incubated at 43C.

The ranking of the plating media was "Choice" best, brilliant green agar next, and brilliant green sulfa agar, the least satisfactory. The differences were statistically significant ($P = 0.02$). The fact that the "Choice" agar was superior to the other two agars is probably an indication of the value of experience in this part of the analysis. Not all laboratories used a "Choice," but each one that did so had the best results with it. Of the laboratories that used a "Choice," no two selected the same agar and in addition, one laboratory indicated their choice to be brilliant green agar and another indicated brilliant green sulfa agar, so additional plates were not used for this test by these two laboratories.

While the use of either the "standard" media supplied from a common lot or local media obtained by the participating laboratories themselves did not significantly affect the ranking (see Table 4) of the methods tried, the standard media did give better recovery than the local media at a significance level of $P = 0.10$. This problem of media variability has been reported for brilliant green agar by Read and Reyes (2). Our results reinforce the need to use common lots of media when attempting to evaluate different methodologies, and further suggest that such lots should be assayed before use to assure that they will perform as expected.

While these experiments have shown a definite advantage in the recovery of *Salmonella* from raw meat by using 43C incubation of the enrichment cultures, the choice of enrichment appears to be somewhat serotype-dependent. Generally best recovery occurred with the use of either selenite brilliant green sulfa or tetrathionate enrichment broths. Experience appears to play a critical role in the use of selective agars. Brilliant green agar appeared more useful as single selective agar for the laboratories participating in this study than did brilliant green sulfa agar or bismuth sulfite agar.

The work should now be extended to test naturally contaminated meats. The stress applied to cells in naturally contaminated meats will vary depending on the handling conditions and age of the product. No laboratory prepared sample can account for all of these, and some may well have a pronounced effect on the ability of a particular method to detect *Salmonella* contamination. Such work should also include a determination of the value of a preenrichment step which has been shown in recent work (1, personal communication*) to be desirable for unprocessed meats.

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*Silliker, J. H., and D. Gabis. Personal communication.

TABLE 4

Ranking of methods based on their ability to detect positive samples (1 = best, 5 = worst)

Source of enrichment	Serotype in sample	BG agar						BG sulfa agar						Choice agar					
		A*	B	C	D	E	P	A	B	C	D	E	P	A	B	C	D	E	P
Standard	*S. typhimurium*	1	2	3	5	3	0.14	2	1	2	4	4	0.31	1	2	4	4	2	0.18
	S. dublin	2	1	2	2	2	0.10	2	1	2	2	2	0.10	2	1	2	2	2	0.10
	S. newport	4	1	2	2	5	—	2	1	2	2	2	0.41	5	2	1	2	4	0.46
	S. worthington	3	1	1	3	3	—	2	2	1	2	2	0.41	2	2	1	2	2	—
	S. senftenberg	3	3	5	1	1	0.03	4	3	5	1	1	0.03	3	4	4	1	1	0.03
Local	*S. typhimurium*	2	1	4	5	3	0.05	2	1	3	3	3	0.08	3	1	4	2	5	0.28
	S. dublin	2	1	2	2	2	0.41	2	1	2	2	2	0.41	2	1	2	2	2	0.41
	S. newport	5	1	3	1	4	—	3	3	1	5	1	—	2	2	1	2	5	—
	S. worthington	1	1	1	5	1	—	2	2	1	2	2	0.41	2	5	1	2	2	0.41
	S. senftenberg	5	3	3	1	1	0.05	5	3	4	1	1	0.05	5	3	4	1	1	0.01

*Enrichments: A, selenite brilliant green sulfa 35C; B, selenite brilliant green sulfa 43C; C, tetrathionate 43C; D, selenite cystine 43C; E, selenite cystine 43C (1/100 dilution when plated). P = probability of more ranks variation than observed. M = P greater than 0.5.

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